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Using Telemetry Data to Refine Endpoints for New Zealand White Rabbits Challenged with *Bacillus anthracis*

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We used a continuous-monitoring digital telemetry system to investigate temperature response in New Zealand White rabbits after inhalation or subcutaneous challenge with *Bacillus anthracis*. Two spore preparations of *B. anthracis* Ames A2084 were evaluated by using a nose-only inhalation model, and 2 strains, *B. anthracis* Ames A2084 and *B. anthracis* UT500, were evaluated in a subcutaneous model. Animal body temperature greater than 3 SD above the mean baseline temperature was considered a significant increase in body temperature (SIBT). All rabbits that exhibited SIBT after challenge by either route of infection or bacterial strain eventually died or were euthanized due to infection, and all rabbits that died or were euthanized due to infection exhibited SIBT during the course of disease. The time at onset of SIBT preceded clinical signs of disease in 94% of the rabbits tested by as long as 2 days. In addition, continuous temperature monitoring facilitated discrimination between the 2 *B. anthracis* strains with regard to the time interval between SIBT and death. These data suggest that for the New Zealand White rabbit anthrax model, SIBT is a reliable indicator of infection, is predictive of experimental outcome in the absence of treatment, and is measurable prior to the appearance of more severe signs of disease. The use of digital telemetry to monitor infectious disease course in animal models of anthrax can potentially be used in conjunction with other clinical score metrics to refine endpoint euthanasia criteria.

Abbreviations: PA, protective antigen; SIBT, significant increase in body temperature; TSA, tryptic soy agar; WFI, water for injection

Biotelemetry is defined as the remote detection and measurement of a human or animal function, activity, or condition (such as heart rate or body temperature). The use of biotelemetry to measure physiologic responses in research animals has evolved dramatically over the last 150 y: from adapting a rubber bulb and tubing to transmit a pneumatic signal from the pectoral muscle of a pigeon in 1869[35] and using abdominally implanted thermocouples to transmit body temperature from guinea pigs through wires to a multiple-point potentiometer in 1967[45] to the modern era of sending digitalized radio signals from implanted transponders capable of measuring a wide variety of physiologic functions simultaneously. With few exceptions (for example, studies on anesthetics), the quality of data collected from conscious animal models is typically considered superior because they better represent the normal state of the animal and are therefore a more predictive model of the correlating human condition.[26] In addition to the improvements in the quality and applicability of the data generated from animal models, biotelemetry offers benefits to animal welfare as well. With biotelemetry, the need for frequent handling of animals and the subsequent use of physical or chemical restraint (and its potential effect on body temperature) needed to obtain physiologic data is greatly reduced, thus eliminating a potentially significant source of stress to the animals. Furthermore, the reduction in animal handling provides a tangible benefit to human safety by decreasing direct contact with animals, which can pose a threat of physical injury to animal care or research staff. This benefit can be even more pronounced when working with animals that have been exposed to infectious agents or other materials that are hazardous to humans.

Biomedical research has taken advantage of this important research tool and used it in diverse species and methodologies to further both human and animal health. Such studies include: evaluating optimal housing conditions for laboratory mice, rats and guinea pigs;[14,37,44] determining physiologic effects of various anesthetics in cynomolgus macaques (*Macaca fascicularis*) and analgesics in rats;[33,44] providing physiologic references for black-tailed prairie dogs (*Cynomys ludovicianus*);[25] testing the efficacy of therapeutic strategies in a rat model of endotoxemia;[11] and assessing drug-induced changes to myocardial contractility in canines,[17] to name only a few.

The incorporation of biotelemetry into the field of infectious disease research has resulted in new insights to pathogenesis and helped to improve the usefulness of animal models, while also resulting in refinements to animal welfare. This has been particularly true in the field of anthrax research. With the ability to collect real-time data on multiple physiologic parameters such as mean arterial pressure, systolic pressure, diastolic pressure, pulse pressure, heart rate with electrocardiogram, respiratory rate, respiratory amplitude, inspiratory time, expiratory time, and temperature, biotelemetry has resulted in new understandings of how physiologic responses to inhalational anthrax in rabbits closely mimics those found in humans,[7,28,48] as well as leading to a better understanding of hemodynamic effects of anthrax toxins in the rabbit model.[29]

Bacillus anthracis is a gram-positive, spore-forming bacillus and the causative agent of anthrax. A Tier 1 biologic select agent,

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B. anthracis has historically been—and remains—a high-priority biologic threat agent. The New Zealand White rabbit model is commonly used to evaluate anthrax disease course and countermeasures by multiple challenge routes.[1,2,6,15,18,27,31,34,36,39,47,48] Recently, 2 separate studies involving the evaluation of *B. anthracis* virulence were conducted at the National Biodefense Analysis and Countermeasures Center. One study used an inhalational route of exposure and the other a subcutaneous route. *B. anthracis* Ames A2084[40] was used for both inhalational and subcutaneous studies, and *B. anthracis* UT500[4,8] was used for the subcutaneous studies. UT500, which to date has been evaluated only in mouse models, was selected to assess its virulence potential in the higher order rabbit model. Both studies incorporated the use of digital telemetry to provide insight into the temperature response of rabbits and to evaluate its potential for establishing a more reliable early endpoint for median lethal dose (LD_{50}) studies.

Anthrax disease course is similar in all animal models tested and follows a characteristic pattern of infection, presence of bacteria and protective antigen (PA) in the blood, and clinical signs of disease, followed shortly thereafter by the animal dying due to infection. From an animal welfare perspective, a primary objective in infectious disease animal studies is to euthanize animals as early in the disease course as possible to minimize pain and distress. This ideal is often difficult to achieve with anthrax models because of rapid late-stage disease kinetics. As such, the use of a reliable and predictive indicator of death would be useful in refining endpoint criteria in anthrax animal models. Here we describe the use of digital telemetry to monitor changes in body temperature as a predictive indicator of death in the rabbit anthrax model.

Materials and Methods

Animals. New Zealand White rabbits (HsdOkd:NZW; weight, 3.4 to 3.7 kg; age, 17 wk at receipt; Harlan, Oakwood, MI) were used to determine median lethal doses and temperature response to infection after exposure to *B. anthracis* by either inhalation or subcutaneous injection. Female rabbits were used for the inhalational challenge studies, and an equal distribution of males and females was used for the subcutaneous challenge studies. All research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).[23] The facility where this research was conducted is fully AAALAC-accredited.

Bacterial strains and growth conditions. *B. anthracis* Ames Ancestor A2084 ($pXO1^+pXO2^+$)[40] and *B. anthracis* UT500 ($pXO1^+pXO2^+$)[4,8] were used for the study. Strain UT500 is a reconstructed strain previously generated by transducing the capsule plasmid (pXO2) from strain 6602 (Pasteur) into strain 7702 (a Sterne-like strain) and was kindly provided by Dr Theresa Koehler (University of Texas Health Science Center, Houston, TX). *B. anthracis* from a single colony was grown in brain–heart infusion broth for 18 h (37 °C, shaking at 200 rpm), followed by the addition of glycerol to a final concentration of 15%. Single-use 1-mL aliquots were prepared in cryovials and stored at –80 °C.

Spore preparations were derived from Leighton Doi[30] and Modified G[22] medium for the inhalational and subcutaneous experiments, respectively. Spore cultures were made by inoculating 200 µL of *B. anthracis* frozen stock into 200 mL sporulation broth in 2-L baffled flasks. These cultures were incubated (37 °C, 200 rpm) for 72 h, or until sporulation reached approximately 95% as determined by phase-contrast microscopy. Two spore preparations were made for the inhalational studies, one with and one without gradient purification. Spores for gradient purification were harvested by centrifugation (JLA 10.5 rotor, 5000 rpm, 20 min, 4 °C; Avanti JE centrifuge, Beckman–Coulter, Brea, CA) in 500-mL centrifuge bottles followed by 2 washes in water for injection (WFI). Gradient purification was performed by gently pipetting 2 mL of spore suspension onto 15 mL of a 58% solution of Omnipaque (Iohexol, GE Healthcare, Silver Spring, MD) in WFI followed by centrifugation in an Avanti JS 5.3 rotor (5300 rpm, 30 min, 4 °C; Beckman–Coulter). The purified spore pellet was washed twice with WFI, resuspended in WFI, and heat-shocked at 65 °C for 40 min; the spore preparation was stored at 4 °C. The unpurified spores were used directly from the sporulation culture and were not heat-shocked prior to inhalation challenge experiments. Spore preparations were enumerated for titer by serial dilution in WFI and plating on tryptic soy agar (TSA). Purified *B. anthracis* Ames A2084 and UT500 spores were used for all subcutaneous challenge experiments.

Telemetry. PhysioTel Digital Model L00 and M00 telemetry transponders (Data Sciences International, St Paul, MN) were used for the inhalational and subcutaneous challenge studies, respectively. Transponders were surgically implanted in the peritoneal cavity of New Zealand White rabbits according to the manufacturer's instructions. Rabbits were sedated by using a combination of ketamine (50 mg/mL, 35 mg/kg IM) and xylazine (10 mg/mL, 5 mg/kg IM) and placed in dorsal recumbency on a warm air circulating pad and maintained on isoflurane (3% for maintenance) in oxygen (1 L/min) delivered by facemask. The ventral abdomen was surgically prepared, and the rabbit was transported to the surgery room, placed back on isoflurane, and underwent a second surgical preparation of the ventral abdomen. The rabbit was covered with a sterile drape, and an approximately 5 cm ventral midline incision made through the skin, fascia, and fat to expose the linea alba. An incision was made through the linea and the body wall was incised to expose the abdominal cavity. The transponder was affixed to the lateral body wall by using 3-0 nonabsorbable suture (Ethibond, Ethicon, Somerville, NJ) and the antennae placed into a subperitoneal tunnel created to maintain it in the optimal position for transmission effectiveness. The body wall was closed by using an inverted cruciate pattern of 3-0 absorbable suture (Monosorb, Henry Schein, Plainville, NY) with a tapered needle. The subcutaneous layer was closed with 4-0 absorbable suture (Monosorb, Henry Schein) on a taper needle by using a simple continuous pattern. The subcuticular layer was closed with 4-0 absorbable suture (Monosorb, Henry Schein) on a cutting needle by using a simple continuous pattern. Perioperatively, rabbits were administered sustained-release buprenorphine subcutaneously (ZooPharm, Laramie, WY) and enrofloxacin (5 mg/kg SC; Henry Schein). Animals were monitored until fully recovered; enrofloxacin was continued twice daily for 5 d and sustained-release buprenorphine was given every 72 h as needed based on clinical signs. Animals were allowed to recover a minimum of 2 wk prior to being placed on a study. After recovery, rabbits were housed in an ABSL3 facility using HEPA-filtered, ventilated, negative-pressure containment cages (Allentown Caging, Allentown, NJ) for at least 7 d before challenge.

Internal body temperatures of rabbits were measured at 10-s intervals. Prior to challenge, baseline temperatures of implanted rabbits were collected for a minimum of 48 h. After the baseline collection period, rabbits were removed from their cages, challenged by 1 of the 2 methods described later, and immediately

returned to the cages for temperature readings, which were continued until the rabbits either died due to infection or were euthanized in accordance with the established clinical endpoint.

Telemetry data analysis. At the end of the study, data were analyzed by averaging the 10-s intervals over 15 min and plotting the corresponding mean temperatures against time. A significant increase in body temperature (SIBT) was defined as the rabbit's mean baseline temperature plus 3 times the mean baseline standard deviation. Time of death was defined as the time of euthanasia or the last activity reading from the implanted transponder which corresponded with a sudden drop in temperature.

Aerosol challenge. Two groups of 10 rabbits each were exposed by inhalation to either gradient-purified or unpurified *B. anthracis* A2084 spore preparations. The target dose was 10 times the estimated median lethal dose. The aerosol inhalation system was housed in a class III biosafety cabinet and consisted of a custom 40-L cylindrical nose-only inhalation chamber and a 3-jet Collision nebulizer (operated at 30 psi; BGI, Waltham, MA) attached to the chamber inlet by means of a 2-in. stainless steel tube. Chamber flow was maintained at 18 L/min by using a mass flow controller (Alicat Scientific, Tucson, AZ) attached to a vacuum pump (model ME8NT, Vacuubrand, Wertheim, Germany). A HEPA filter (TSI, Shoreview, MN) attached just downstream of the nebulizer allowed clean air for dilution to be drawn into the system. The aerosol concentration in the chamber was measured by using 25-mm gelatin filters (PN 225- 9551, SKC, Eighty Four, PA) operating at 1 L/min and located in the breathing zone of the test subject.

Prior to each test, a 10-mL aliquot of *B. anthracis* spores was transferred to the nebulizer. A rabbit was placed in a restrainer to which it had been previously acclimated (for 10 min at least 1 d prior to aerosol exposure) and transferred to the class III Biosafety Cabinet containing the inhalation exposure system. The nose of the rabbit was placed into a small opening in a sheet of latex dental dam (0.010-in. Abrasion-Resistant Natural Latex Rubber, McMaster Carr, Robbinsville, NJ) located on the side of the chamber. The nebulizer was then activated and aerosol generated for 10 min. The aerosol concentration was allowed to decay for an additional 5 min before the rabbit was removed and returned to a primary isolator cage in an animal holding room. Gelatin filter samplers continued sampling during the 5-min decay period, after which the filter sampler flow was stopped. The filters were removed from the chamber, dissolved in PBS at 37 °C, serially diluted, and spread plated on TSA to estimate the amount of viable *B. anthracis* collected. The results of the plating assay (in cfu) were used to determine the average aerosol concentration in the test chamber during the sampling period and the inhaled dose received by each subject. The average aerosol concentration was estimated as the total number of cfu collected divided by the total amount of air sampled. The inhaled dose was calculated as the product of the average aerosol concentration, the respiratory minute volume estimated from the body weight of the test subject, and the exposure duration.[3]

The particle size distribution of the generated aerosols was determined in separate tests without rabbits present by using Mercer cascade impactors (3.5 L/min; InTox Products, Moriarty, NM) located inside the exposure chamber; a tube was inserted in the dental dam opening to block airflow and serve as a phantom in the aerosol chamber. After termination of aerosol generation, the impactor was removed from the chamber and each stainless steel stage of the impactor was placed into a 50-mL conical tube containing 10 mL PBS and vortexed briefly (10 s) to resuspend the bacteria collected on the stage. Serial dilutions of the resuspended material were spread on TSA to estimate the amount of viable agent present on each stage, expressed as cfu. The mass median aerodynamic diameter and geometric standard deviation were determined from the results of the plating assay by using a previously described method.[21]

Subcutaneous challenge. To evaluate the effect of *B. anthracis* strain on disease course, 2 groups of 10 rabbits each, surgically implanted with digital transponders, were challenged by subcutaneous injection with a target dose of 1000 cfu of either *B. anthracis* Ames A2084 or UT500 spores. The LD_{50} of each strain was determined by administering escalating doses of purified spores to groups of an equal distribution of male and female rabbits. Telemetry was not used for the LD_{50} study. For subcutaneous injections, rabbits were brought to the front of the cage by using a squeeze-back mechanism and injected subcutaneously in the left shoulder with 500 µL of spore suspension in WFI. The actual doses for each group were determined by serial dilution of each spore challenge dose and plating on TSA.

Clinical observations. After exposure, rabbits were monitored for clinical signs of infection twice daily for 10 d. Animals showing signs of infection were monitored 3 times daily and were euthanized when they met criteria on a predetermined clinical scoring sheet with 3 categories: appearance, natural behavior, and provoked behavior. Clinical signs in each category were assigned a numerical score starting with 0 (normal) and increasing in the level of severity. Examples of clinical signs for scores in each category included: appearance (1, dull or rough coat; 2, droopy ears or ocular/nasal discharge; 3, hunched posture or abdominal breathing); natural behavior (1, reduced interaction; 2, decreased appetite or fecal output or lethargy; 4, vocalization or head pressing); provoked behavior (1, subdued/normal when stimulated; 2, subdued when stimulated; 4, unresponsive when stimulated, weak or precomatose). The scores best representing the clinical condition in each category were summed to represent the total score. When the total score was 3 to 6, the frequency of observations was increased, and rabbits were euthanized when the total score was equal to 7 or greater.

Biologic samples. Blood samples were collected by cardiocentesis from rabbits that died or were euthanized due to infection, or were euthanized at the end of the study. The blood was collected in EDTA tubes, serially diluted in PBS, and plated on TSA to determine the terminal *B. anthracis* concentration (cfu/mL) in the blood. To obtain serum, blood was collected in serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged in a JS 5.3 rotor (1000 rpm, room temperature) and the serum stored at -20 °C until use. Serum samples were serially diluted in PBS and the PA concentration measured with a quantitative PA ELISA kit (Alpha Diagnostics International, San Antonio, TX).

In addition, spleen samples were collected from rabbits that died or were euthanized due to infection or at the end of the study. Pieces of spleen were placed in a preweighed 50-mL conical tube and stored at approximately 4 °C prior to processing, typically no more than 2 h. Once the mass of spleen (0.5 to 1 g) was recorded, a sterile 70-µm filter basket was placed in a 50-mL conical tube and the spleen pieces placed in the basket. PBS (2 mL) was added to the filter basket to rinse the sample, and a 5-mL syringe plunger was used to macerate and mix the spleen with the PBS diluent. The filtered spleen effluent (typically about 8 mL) was collected in the conical tube, serially diluted in PBS, and plated on TSA to determine the bacterial load (in cfu/g) in the spleen.

Statistical analysis. Subcutaneous LD_{50} values were calculated from the mortality data by using probit LD_{50} analysis.[13] The

results from both the aerosol and subcutaneous exposure groups were compared by using a 2-tailed Fisher Exact Test (Prism version 5.04 for Windows, GraphPad Software, San Diego CA). The test was conducted separately for each exposure route by using the pooled results of both aerosol exposure days (half of the aerosol challenge groups were exposed on each of 2 successive days), with the nominal variables being the presence or absence of fever and the death or survival of the test subjects. For binomial analysis, the Clopper–Pearson method was used to calculate confidence intervals for the probability of death given a positive SIBT result (MatLab, Natick, MA). Survival curves were compared by using Kaplan–Meier log-rank analysis.[38] Data distribution was evaluated according to the Shapiro–Wilk normality test.[43] Results were considered statistically significant when the P value was less than 0.05.

Results

Temperature response to challenge with aerosolized *B. anthracis*. Among the 20 rabbits surgically implanted with digital transponders and challenged with approximately 1×10^6 aerosolized purified or unpurified *B. anthracis* Ames A2084 spores, 18 (purified spores, $n = 8$; unpurified spores, $n = 10$) died or were euthanized due to infection. The mass median aerodynamic diameter particle size of the purified spores was 1.4 µm with a geometric standard deviation of 1.4 µm whereas for unpurified spores, the respective values were 3.0 and 1.4 µm. All rabbits that died or were euthanized due to infection, regardless of spore preparation, exhibited SIBT approximately 30 to 40 h after aerosol exposure. In addition, all rabbits that exhibited SIBT eventually died or were euthanized due to infection, between 2 and 35 h after onset of SIBT, with a mean time of approximately 17 h between SIBT and death (Figure 1). Of the 8 animals that died from infection with purified spores, 6 were found dead and 2 were euthanized. Of the 10 animals that died from infection with unpurified spores, 7 were found dead and 3 were euthanized. All animals that died or were euthanized had bacteremia which ranged from 1.83×10^2 to 3.1×10^9 cfu/mL, indicating that death was attributable to anthrax infection. None of the control rabbits (implanted with transponders and challenged with vehicle [WFI] only) or survivors exhibited SIBT during the observation period or were bacteremic at the end of the study.

Temperature response to subcutaneous challenge with *B. anthracis*. To evaluate the effect of *B. anthracis* strain on disease course, groups of 10 rabbits surgically implanted with digital transponders were challenged subcutaneously with a target dose of 1000 cfu of either Ames A2084 or UT500 purified spores. The actual challenge doses were 1433 and 1083 cfu for Ames A2084 and UT500, respectively. As observed for the inhalational model, all animals that died or were euthanized after subcutaneous infection with either Ames A2084 ($n = 10$) or UT500 ($n = 7$) developed SIBT, and all animals that developed SIBT eventually died or were euthanized due to infection. Of the 10 animals that died from infection with Ames A2084, 7 were found dead and 3 were euthanized. Of the 7 animals that died from infection with UT500, 5 were found dead and 2 were euthanized.

Temperature responses in rabbits challenged with Ames A2084 were markedly different from those of animals challenged with UT500. Challenge with Ames A2084 resulted in a single spike in temperature that was followed shortly thereafter by death or euthanasia due to infection. In contrast, rabbits challenged with UT500 exhibited SIBT, which subsequently returned to baseline temperature, followed by multiple SIBT and eventual death or euthanasia due to infection (Figure 2). Survival curve

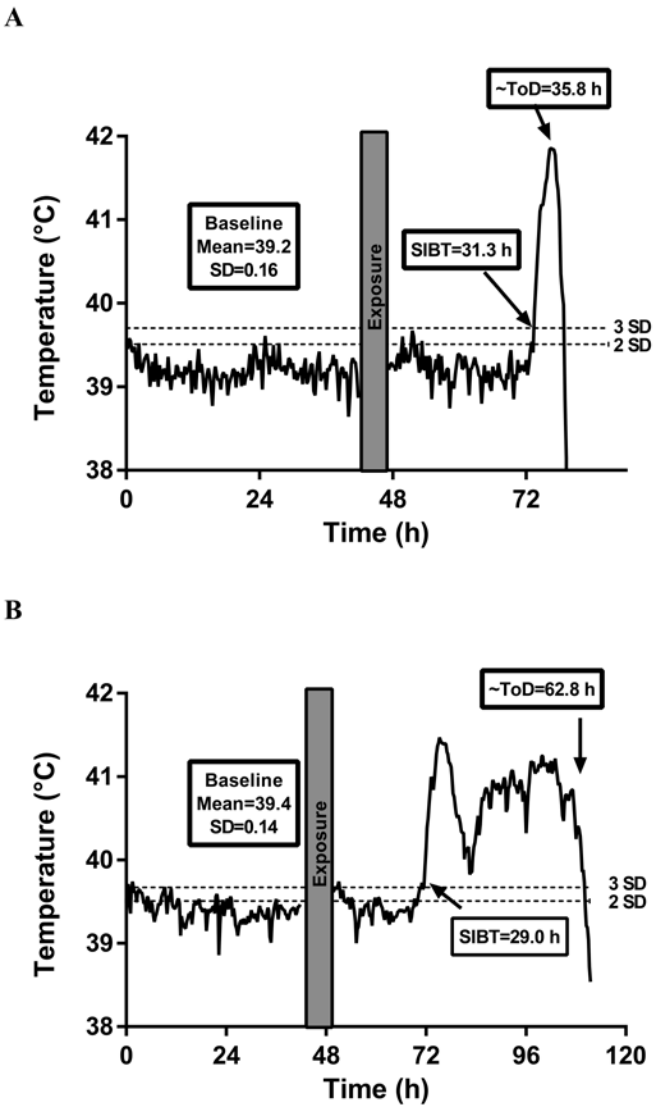

Figure 1. Temperature response after inhalational exposure to *B. anthracis* Ames A2084 spores. Shown are 2 characteristic responses of (A) significant increase in body temperature (SIBT) leading to death shortly thereafter and (B) SIBT followed by a recovery period and then death. SIBT is defined as a body temperature that is 3 SD above the mean baseline temperature. Mean baseline temperature and SD are indicated. Arrows indicate time of SIBT and death (ToD). The gray bar indicates the time of challenge. Measurement of baseline temperature began at T0 and continued for several days prior to challenge.

analysis indicated that SIBT in animals challenged with UT500 occurred slightly later ($P = 0.036$) and lasted longer ($P = 3 \times 10^{-6}$) than in rabbits challenged with Ames A2084 (Figure 3, Table 1). In addition, terminal bacterial counts in the blood and spleen were lower ($P = 0.006$ and $P = 0.0006$, respectively) in animals infected with UT500 compared with Ames A2084 (Figure 4 A). Similarly, corresponding terminal serum PA concentrations were lower ($P = 0.003$) in animals infected with UT500 (Figure 4 B). There was no effect of sex or dose on resulting bacterial burden or serum PA concentration for either *B. anthracis* Ames A2084 or UT500.

Exposure to either *B. anthracis* Ames A2084 (inhalational or subcutaneous) or UT500 (subcutaneous) resulted in significant differences in survival between rabbits that exhibited SIBT and those that did not ($P = 0.002$ and $P = 0.008$, respectively, Fisher Exact Test). Across all data for animals exposed to *B. anthracis*

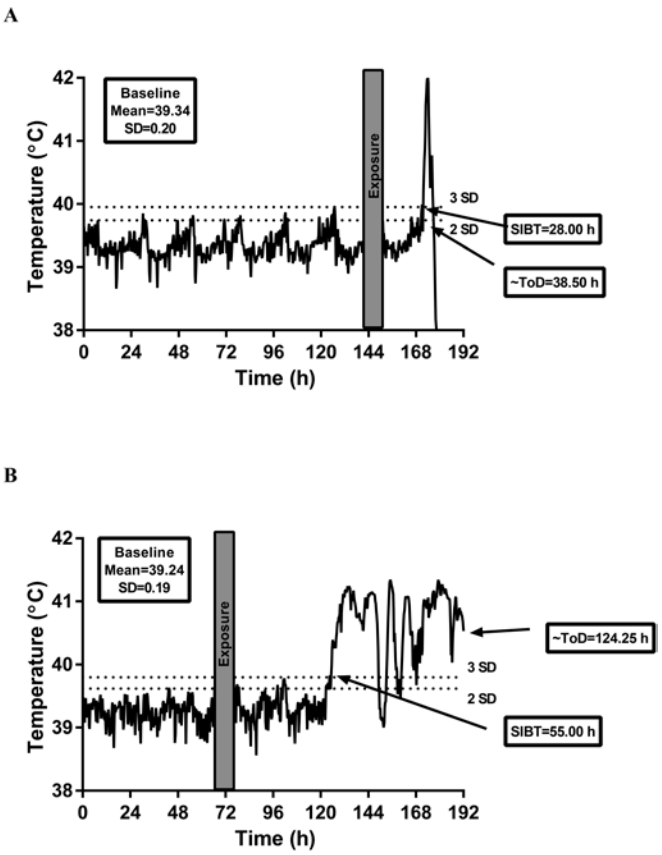

Figure 2. (A) Single-spike SIBT response in a female rabbit challenged subcutaneously with *B. anthracis* Ames A2084 (B) multiple SIBT in a female rabbit challenged subcutaneously with *B. anthracis* UT500. Arrows indicate time of SIBT and death (ToD). The gray bar shows time of challenge. Measurement of baseline temperature began at T0 and continued for several days prior to challenge.

Ames A2084 by inhalation or subcutaneous challenge, the 95% CI for the probability of death after SIBT were 0.88 and 1.0. The corresponding values for rabbits exposed to either *B. anthracis* Ames A2084 or UT500 were 0.90 and 1.0, respectively. Therefore, a fever response in New Zealand White rabbits after challenge with *B. anthracis* spores is strongly predictive of mortality (Table 2).

SIBT response and clinical score. The data from the inhalational and subcutaneous challenge studies suggested that SIBT is an early and predictive indicator of terminal infection after challenge with *B. anthracis*. To evaluate SIBT compared with the clinical score as an indicator of infection, the times between challenge and either SIBT or a clinical score greater than 0 were compared. Of the 33 recorded SIBT, 31 occurred prior to the first clinical score. The only incidents of a clinical score that preceded SIBT were from 2 animals in the inhalational study; these animals received a score of 1 or 2 prior to SIBT. The mean time between SIBT and the first clinical score after inhalational or subcutaneous challenge with *B. anthracis* Ames A2084 was 16.5 ± 12.2 h and 16.7 ± 12.0 h, respectively. The mean time after subcutaneous challenge with *B. anthracis* UT500 was 52.6 ± 31.8 h.

UT500 virulence and sex bias. To examine the relative virulence of *B. anthracis* Ames A2084 and UT500, spores of each strain were prepared by using Modified G medium. Five groups of 7 rabbits each were challenged through subcutaneous injection with purified spores. Mortality and time to death after challenge was tabulated and used to calculate LD_{50} values and construct survival curves for each strain. Times between challenge

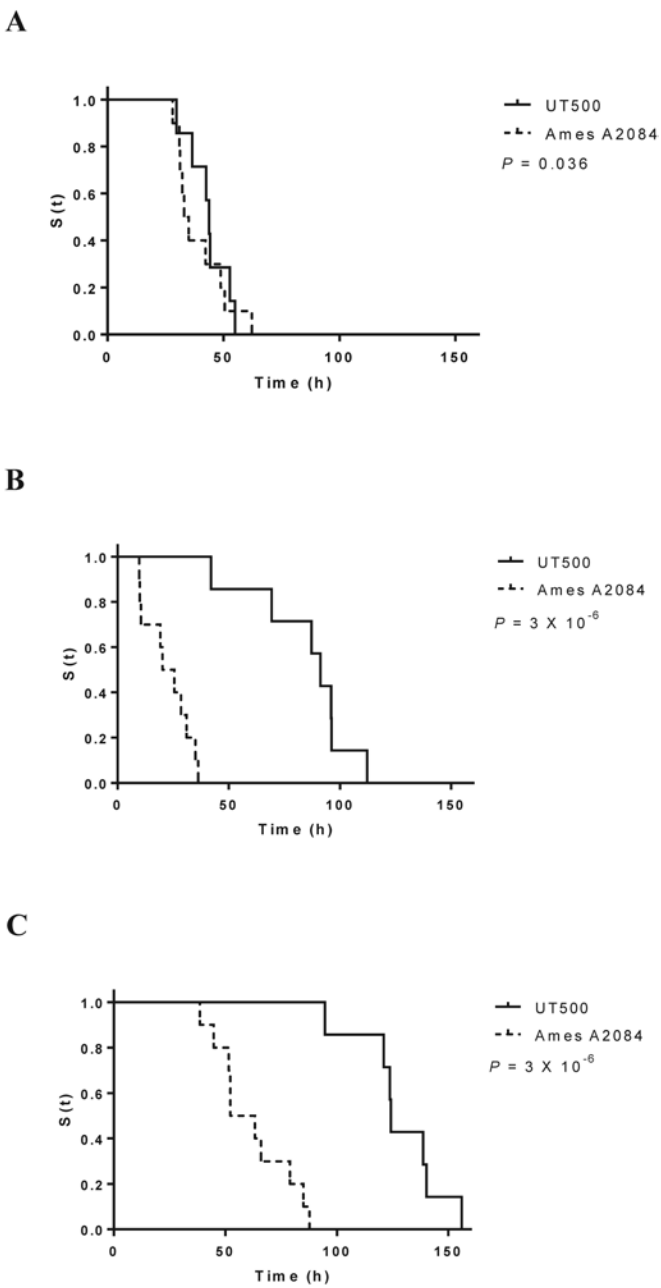

Figure 3. Survival curve analysis of rabbits challenged subcutaneously with *B. anthracis* Ames A2084 (5 males and 5 females; challenge dose 1433 cfu) compared with *B. anthracis* UT500 (5 males and 5 females; challenge dose 1083 cfu). Times plotted were from (A) challenge to SIBT, (B) SIBT to death, and (C) challenge to death. Solid lines show response after challenge with UT500; dashed lines indicate response after challenge with Ames A2084. All 3 parameters showed significant (Mantel–Haenszel log-rank test) interstrain differences.

and death were longer ($P = 2 \times 10^{-8}$) in animals infected with UT500 compared with Ames A2084, consistent with the telemetry study. The LD_{50} of Ames A2084 was 7 cfu (95% CI, 1 to 35) and that of UT500 was 60 cfu (95% CI, 9 to 413); these values do not differ significantly. In addition, the LD_{50} 84% confidence intervals likewise overlapped and thus did not meet statistical significance. An unexpected finding from this study was that female rabbits were significantly more susceptible to *B. anthracis* UT500 than male rabbits. By combining the data from the dose response and telemetry studies, an LD_{50} could be calculated for female and male rabbits. The LD_{50} of UT500 in female rabbits

Table 1. Disease course in rabbits that died or were euthanized due to infection with *B. anthracis* Ames A2084 or UT500 after subcutaneous challenge

Parameter	Time (h; mean ± 1 SD)		P^a
	Ames A2084	UT500	
Challenge to SIBT	39.4 ± 11.2	43.5 ± 8.7	0.036
SIBT to death	22.6 ± 10.2	84.9 ± 22.8	3×10^{-6}
Challenge to death	62.0 ± 17.2	128.4 ± 19.3	3×10^{-6}

Ten animals (5 males and 5 females) were challenged with each strain. All 10 rabbits challenged with Ames A2084 died or were euthanized due to infection, and 7 (2 males and 5 females) of the 10 animals challenged with UT500 died or were euthanized due to infection.
[a]Mantel–Haenszel log-rank test

was 4 cfu (95% CI, 0.3 to 63) and that for males was 1846 cfu (95% CI, 64 to 5.2×10^4). These data contrast with values of 4 cfu (95% CI, 1 to 25) and 18 cfu (95% CI, 2 to 147) for *B. anthracis* Ames A2084 in female compared with male rabbits, respectively. The 95% CI of the female and male UT500 LD_{50} values do not overlap, indicating a statistically significant sex-associated effect on virulence. The nonoverlapping 84% CI, which equate to a significance level of approximately 0.05,[24] were 1 to 30 for female rabbits compared with 168 to 2.0×10^4 for male rabbits. In contrast, the corresponding Ames A2084 LD_{50} 84% CI were 1 to 15 compared with 4 to 82) for female and male rabbits, respectively. Between the LD_{50} and telemetry studies, the total number of animals exposed to each strain was 45 for *B. anthracis* Ames A2084 (24 females, 21 males) and 45 for *B. anthracis* UT500 (23 females, 22 males). Overall 5 of 24 female rabbits and 7 of 21 male rabbits survived challenge with Ames A2084. By contrast, 4 of 23 female rabbits and 16 of 22 male rabbits survived challenge with UT500 (Table 3).

Samples of blood (female, n = 13; male, n = 4) and spleen (female, n = 12; male, n = 4) were collected from rabbits that died or were euthanized due to infection. The terminal bacterial burden in blood (P = 0.98, unpaired T-test) or spleen (P = 0.79, unpaired T-test) did not differ between female and male rabbits infected with UT500. All rabbits that exhibited SIBT eventually died or were euthanized to infection, whereas none of the animals that survived to the study endpoint exhibited SIBT.

Discussion

In this report, we describe the use of digital telemetry to monitor anthrax disease course and temperature response in New Zealand White rabbits and demonstrate that SIBT is a reliable indicator of experimental outcome in untreated animals. Rabbits are a well-established model of *B. anthracis* infection, but the short clinical course and relative scarcity of outward clinical signs seen with this model complicate the ability to establish an early endpoint for intervention to eliminate or minimize pain and distress. Although the use of a clinical scoring checklist with intervention criteria helps to reduce the prolongation of pain and distress, it is not highly effective in identifying animals early in the disease course for the reasons just mentioned.

In the current study using the New Zealand White rabbit model of anthrax, we observed a 100% correlation between the development of SIBT and death from infection in untreated animals. This correlation was true for 2 routes of infection (inhalational and subcutaneous), 2 *B. anthracis* spore preparations (purified and unpurified), and the 2 strains tested (*B. anthracis* Ames A2084 and *B. anthracis* UT500). In additional ongoing studies with New Zealand White rabbits exposed to *B. anthracis* Ames A2084, we have noted the same high correlation for an additional 39 animals that died or were euthanized due to infection and 41 animals that survived (equal distribution of males and females). All animals that died or were euthanized exhibited

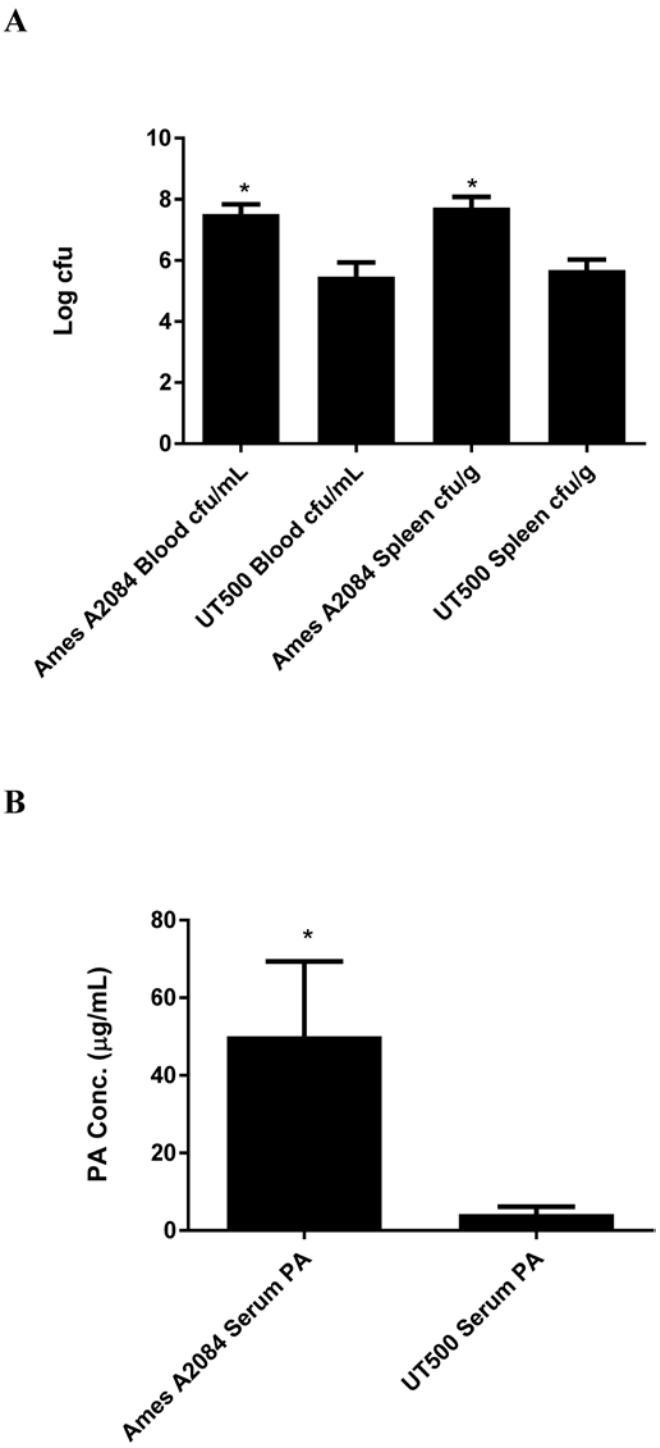

Figure 4. Log-transformed values of terminal bacterial burden in the blood and spleen and terminal PA concentration in the serum. Bacterial burden and serum PA concentrations were derived from terminal blood and spleen samples collected from dose groups during the subcutaneous LD_{50} studies for *B. anthracis* Ames A2084 (blood and spleen: n = 9 [4 males, 5 females]) and UT500 (blood: n = 17 [4 males, 13 females]; spleen: n = 16 [4 males and 12 females]). Ames A2084 doses ranged from 2 to 1433 cfu; UT500 doses ranged from 3 to 1300 cfu. (A) Blood and splenic bacterial burdens differed significantly (*; P = 0.006 and 0.0006, respectively, unpaired t test) between Ames A2084 and UT500. (B) Serum PA concentrations differed significantly (*; P = 0.003, unpaired t test), between Ames A2084 and UT500.

Table 2. Correlation between SIBT and survival

Challenge route	Strain	Total no. of rabbits	No. with SIBT[a]	No. that died	No. survived[b]
Subcutaneous	Ames A2084	10	10	10	0
	UT500	10	7	7	3
	Vehicle control	2	0	0	2
Inhalation	Ames A2084 purified spores	10	8	8	2
	Ames A2084 unpurified spores	10	10	10	0
	Vehicle control	2	0	0	2

[a]All rabbits that exhibited SIBT eventually died or were euthanized due to infection.
[b]None of the surviving animals previously exhibited SIBT.

Table 3. Summary of disease course in rabbits challenged subcutaneously with *B. anthracis* Ames A2084 or UT500

	Dose	Sex (no. that died/total no. challenged)	Time (h, mean ± 1 SD) after challenge until death
Ames A2084			
	1433[a]	Female (5/5)	56.6 ± 12.3
		Male (5/5)	74.8 ± 21.4
	840	Female (4/4)	71.8 ± 14.3
		Male (2/3)	79.3 ± 4.6
	144	Female (3/3)	65.7 ± 19.2
		Male (3/4)	49.0 ± 6.9
	31	Female (3/4)	74.3 ± 4.6
		Male (2/3)	60.0 ± 13.4
	8	Female (2/4)	61.0 ± 21.2
		Male (2/3)	94.0 ± 0.0
	2	Female (2/4)	81.3 ± 16.6
		Male (0/3)	N/A
UT500			
	1300	Female (4/4)	136.9 ± 36.1
		Male (2/3)	86.0 ± 12.7
	1083[a]	Female (5/5)	130.0 ± 26.5
		Male (2/5)	133.0 ± 12.7
	300	Female (3/3)	90.3 ± 7.2
		Male (1/4)	167.0
	82	Female (3/4)	120.0 ± 21.1
		Male (0/3)	N/A
	13	Female (2/4)	142.8 ± 34.3
		Male (1/3)	167.0
	3	Female (2/3)	136.0 ± 17.0
		Male (0/4)	N/A

N/A, not applicable (all animals survived)
[a]From telemetry study

SIBT, and all animals that survived did not exhibit SIBT (data not shown). Because of these results, we have incorporated SIBT into our clinical score sheet for this infection model. The data suggest that in New Zealand White rabbits, SIBT precedes signs of anthrax disease and may be an indicator of terminal infection prior to the animal experiencing pain or distress that is not relieved with appropriate and adequate anesthetics, analgesics, or tranquilizer drugs (USDA pain and distress category E). Based on these data, we suggest that, for anthrax studies using New Zealand White rabbits for which death is a critical data point and therapeutics are withheld (for example LD_{50} determinations), SIBT should be included as an endpoint indicator in conjunction with a clinical checklist to set predetermined criteria for humane euthanasia. This approach can be considered a refinement of existing clinical scoring criteria that use animal appearance and behavior to determine endpoints for humane euthanasia intervention, especially because progression to death is rapid after the onset of visually observable clinical signs in this model. Although continuous temperature monitoring currently requires surgical implantation of telemetry transponders, animals typically heal and recover in several days. In addition, with improvements in technology, it is possible that continuous temperature monitoring may be feasible in the future with devices, similar to those used for radiofrequency identification, which can be injected into the subcutaneous tissue, potentially bypassing the requirement for surgical implantation.

In addition, other specific markers, including bacteremia and the presence of toxin in the blood, can potentially be used in establishing clinical endpoint criteria. In this study, we did not perform serial bleeds to correlate these factors with temperature response. We did, however, observe a large range of terminal bacteremia levels in animals challenged with *B. anthracis* Ames A2084, although in 70% of the blood samples tested, the terminal titer was 1×10^7 cfu/mL or greater. The cause of these ranges is unknown but may be related to death due to bacteremia, toxemia, or a combination of the 2. Terminal bacteremia levels in animals infected with *B. anthracis* Ames A2084 or UT500 did not influence euthanasia criteria given that we have not observed any correlation between terminal bacterial titers and clinical score progression. Infected rabbits typically did not score greater than a 1 or 2 until the last few hours before death. As such, and as is typical with anthrax infection in this model, many of the animals were found dead, even though we observed them at least 3 times daily. The use of an earlier endpoint could thus dramatically reduce the occurrence of pain and distress in natural history and LD_{50} studies in which the prediction of death is a critical component. Previous studies have demonstrated that, in rabbit models of anthrax infection, an increase in body temperature after infection correlates with bacteremia and serum PA concentration and could be used as a marker in time-to-treatment studies.[7,28,48] Although such nonhost-specific markers can be predictive of death and could potentially be used in combination with SIBT to refine clinical endpoints, their use would require blood sampling at regular and relatively short intervals, given that the time interval between detection of these markers and death is relatively brief. In addition, such methods increase personnel risk, particularly when animals are exposed to infectious agents. By contrast, digital telemetry provides a continuous readout that potentially can be used to dramatically reduce times between detection and veterinary intervention and that does not require animal handling.

In addition to correlating temperature response with the experimental outcome, using digital telemetry allowed comparisons between *B. anthracis* spore preparation methods, route of exposure, and the *B. anthracis* strain tested. We observed longer times between SIBT and death as well as lower terminal bacteremia and serum PA concentrations in animals challenged with UT500 compared with Ames A2084. The reasons for these differences are not evident since UT500 appears phenotypically identical to Ames A2084. Furthermore, we were unable to identify possible targets from a whole-genome sequence comparison, except for an alanine-to-serine transition in the UT500 lethal factor allele at amino acid 299, commonly found in Sterne but not Ames strains.[40] The disease course in rabbits infected with UT500 revealed an interesting similarity with that observed for edema factor or lethal factor mutants administered by intravenous[34] or subcutaneous injection[32] in rabbits, in which mean time to death was longer and bacterial burdens lower for the single-allele mutants. Given these similarities, a possible hypothesis is that either expression or enzymatic activity of either edema factor or lethal factor is lower in UT500 than Ames a2084. We are currently exploring these possibilities. Taken together, our current data support the use of the rabbit anthrax model when evaluating genetically reconstructed strains and when assessing strain dependent anthrax pathogenesis.

Sex-related differences in human infectious diseases have been reported, with females generally being more resistant than males.[16] With regard to anthrax infection, an analysis of mortality records from 9 documented naturally occurring anthrax outbreaks in bison between 1962 and 1993 revealed a substantially higher proportion of deaths among males,[10] and a recent report described a differential sex-associated response in some strains of mice infected with *B. anthracis* Ames, in which female mice survived significantly longer than males and required a higher spore challenge for mortality.[46] However, experiments with unvaccinated guinea pigs, rabbits, and rhesus macaques have not demonstrated sex-associated differences after spore challenge.[12] The results we present here suggest that female New Zealand White rabbits may be more susceptible than male rabbits to parenteral challenge with UT500, with calculated LD_{50} values of 4 (95% CI, 0.3 to 63) and 1846 (95%CI, 64 to 5.22×10^4), respectively. Note that animals challenged with Ames A2084 did not show a sex-associated effect, given that the calculated LD_{50} values did not differ significantly between females and males. To our knowledge, the present study represents the first report of a sex-associated bias in a rabbit model of anthrax infection. The reasons for this observation are not evident, given that the male and female rabbits were of similar weight (3.4 to 3.7 kg) and age (17 wk) at receipt, and there was no apparent survival trend based on size. Elucidation of the mechanism for this bias requires further investigation and may reflect multiple factors. Assuming that SIBT is an early indicator of dissemination from the site of infection and progression to a systemic disease and because mortality did not differ between male and female rabbits that exhibited SIBT, the data suggest that the higher resistance in male rabbits may be associated with inhibition of this initial dissemination. To date, UT500 has only been examined in mouse models using female mice. To evaluate a possible sex-associated effect of UT500 in mice, experiments could be performed with male mice and compared with previous studies that evaluated UT500 in female mice.[5,9,19]

Although the results reported here suggest a high correlation between a fever response and mortality in New Zealand White rabbits infected with *B. anthracis*, fever is not an effective predictor of death for all species used for anthrax research. Studies conducted with African green monkeys showed that a febrile response occurred inconsistently after inhalational challenge, and only half of the animals that died had developed a fever according to telemetry data.[41] Telemetry has also been used to monitor body temperature of guinea pigs in an anthrax inhalational challenge model.[42] Abnormal temperature readings were observed in these studies, but the increase in temperature was not as clearly identifiable as in the New Zealand White rabbit model. In addition, temperature response did not strictly correlate with infection in a cynomolgus macaque anthrax model.[20] Taken together, the current data suggest that the New Zealand White rabbit anthrax model is highly suitable and superior to other anthrax animal models for implementing SIBT in clinical endpoint euthanasia criteria. SIBT as an endpoint indicator would primarily be useful in studies for which countermeasures are not tested, because vaccinated or treated animals can exhibit bacteremia after anthrax challenge but eventually recover. As noted earlier, SIBT can be used as a trigger for initiating antibiotic treatment.[7,48] However, even in such studies, SIBT can be included in a clinical scoring checklist as a reliable indicator of infection.

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